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TOXICITY EVALUATION OF RASA KARPOORA KULIGAI IN WISTAR RATS

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ABSTRACT

Evaluation of toxicity is important before starting clinical trial of any drug. Need for traditional medicines have been increasing in the recent past, so it is necessary to ensure that the medicine prescribed are safe. Aim of the study is to evaluate the safety profile of Rasa Karpoora Kuligai (RKK) through Acute Oral Toxicity study and 28-Day Repeated Dose Oral Toxicity Study as per OECD guidelines. In Acute study, Animals were divided into 3 groups. Group I served as a control and treated with water. The remaining two groups were treated with 300mg/kg.b.wt and 2000mg/kg.bwt of Rasa Karpoora Kuligai orally. Animals were observed for toxic signs for 14 days and gross pathology was performed at the end of the study. In repeated dose toxicity study, the animals were divided into four groups. The first group was treated as control and second, third, fourth groups were treated with Low dose 230mg/kg/ b.wt, Mid dose 450mg/kg/b.wt, High dose 600mg/ kg/ b.wt of Rasakarpoorakuligai. In acute oral toxicity study, no treatment related death or toxic signs were observed. The 28-day repeated dose study did not show evidence of any significant treatment related changes in all observations from low dose to the high dose level, when compared with the control. Histopathological examination also revealed that no abnormalities. This study ensures the safety of the drug.

KEYWORDS: Rasakarpoorakuligai, Toxicity, OECD guidelines.

INTRODUCTION

Siddha System of Medicine is believed to be originated from lord shiva the supreme god of Tamil and, he is considered to be the principal siddhar. Lord shiva preached this grateful science to shakthi, the goddess and the nanthi from them the siddha system was made available to common people by siddhars. Siddhars were those who were not only physicians but also social reformers. They were well versed in the field of medicine, natural science, alchemy, astrology, etc. Siddhars were the persons who attained siddhi, that perfection and who had overcome death through these Siddha medicines. They give many excellent medicines which cure many challengeable diseases like diabetes, TB, HIV & Cancer.

It is estimated that at some point during lifetime approximately 39.6% of women and men will be diagnosed of cancer. In India, it is estimated that 14.5 lakh people are living with the disease, with over 7 lakh new cases being registered every year and 5,56,400 deaths which are said to be cancer related. An estimated 71% of cancer related deaths are occurring in the age group between 30 and 69 years.[1]

As rights said in this modern world the air we breathe, the food we eat and our lifestyle are all carcinogenic and are ultimately leading us by one way or other to dreadful disease cancer. Everyone in this world rich or poor, men or woman, young or old, and even animals or prone to affect by this disease and that could be prophylactically given against this disease. Also, there is no medicine in the world that good completely cure this disease.

The Currently available treatments for Cancer viz Radiation, Chemotherapy and Surgery are all very Limited Value. The Surgical Procedures are useful only is very early stage and it is miserable that the disease is often detected in advanced stage. The Radiation technique and Chemotherapy are useful only to prolong the survival period.[2] In the pathetic Situation when scientist all over the world struggling to formulate a new medicine to combat the disease. In our Siddha system of medicine, we had thousands of medicines with the indications to cure the disease.

Among various medicine mentioned to treat cancer in Siddha literature. Rasa Karpoora Kuligai is one of the Siddha herbomineral formulation mentioned to treat various type of cancer.[3] Rasa Karpoora kuligai shows significant anti-oxidant and anticancer effect against HeLa cell lines studied by in vitro (V.Manjari, et.al, 2016, WJPR, Vol-v, Issue-9). The safety profile of Rasa Karpoora Kuligai is not scientifically validated. Safety profile of Rasa

Karpoora Kuligai (RKK) through acute and 28 days repeated oral toxicity studies was done as per OECD guideline 423 and 407.

Procurement and Authentication of Raw Drugs

Calomel (*Pooram*) was purchased from local market in Chennai. It was authenticated at Department of Gunapadam in National Institute of Siddha, Chennai. The plants *Piper betle*, *Piper nigrum* and *Allium sativum* were authenticated by Botanist, National Institute of Siddha, Chennai.

Preparation of Rasakarpoora kuligai

The raw ingredients were purified as mentioned in Siddha literature. Rasakarpoora kuligai was prepared using the procedure described in Siddha literature. The drug dose is 0.798 mg (i.e., *Sundai alavu*).

Animal Care and Husbandry

The study protocol involving animals was reviewed and approved by Institutional Animal Ethical Committee (IAEC), National Institute of Siddha, Chennai, India, with the IAEC approval No: NIS/IAEC/13/2016, dated 29.9.2016, prior to the conduction of the study. Experiments were performed as per OECD guidelines. Male and female Wistar albino rats, (130–160 g) obtained from authorized animal breeders of the animal laboratory in TANUVAS, Madhavaram, Chennai and stocked in the animal house at National Institute of Siddha, Chennai. Animals were acclimatized for seven days prior to do toxicity study.

Acute Oral Toxicity Study

Female wistar Albino Rats of weighing 150-200g were used for acute toxicity study. Animals were housed in a cage at 22°C ±3°C and relative humidity 30–70% and have free access to standard rat pellet diet. After seven days of acclimatization the animals were divided into three groups randomly (Group I, II & III). Each group contains 3 female wistar albino rats. Group I served as a control and treated with water. The remaining two groups were treated with 300mg/kg.b.wt and 2000mg/kg.bwt dosage of Rasa Karpoora Kuligai by oral route after 12 hrs fasting with free from water. After drug administration behavioural parameters were monitored for the first 4 hours continuously (1/2 hr, 1hr, 2 hr, 3 hr, 4 hr) and recorded. Then the animals were observed once daily for further 14 days for any mortality and morbidity. Animals were weighed and sacrificed under the intra peritoneal injection of Pentothal Sodium

on the 15th day of the Study period. The toxicological effect was assessed based on mortality.[4]

Repeated Dose Oral Toxicity

150-200 gms of 20 male and 20 female wistar albino rats were used for 28 days repeated oral toxicity study. The animals were divided into four groups. Each group contains 10 animals (5 Female and 5 Male). The first group treated as control and second, third, fourth groups were treated with Low dose 230mg/kg/ b.wt, Mid dose 450mg/kg/b.wt, High dose 600mg/ kg/ b.wt of Rasakarpoorakuligai mixed with twin80 and RO water for 28 days respectively after 12 hrs of fasting with free from water. The low dose, mid dose and high dose of test drug were calculated from human therapeutic dose based on by using the conversion table Paget and Barnes 1964. The control animals were administered with twin 80 mixed RO water. The administration was given by oral, once daily for 28 consecutive days. The animals were observed the behavioural parameters for the study period. Body weight of the animal was being monitored at weekly intervals. Food & water intake were Calculated daily. All the animals were sacrificed at the end of the study (29th day) by using the intra peritoneal injection of Pentothal Sodium as prescribed dose level. Blood was collected from the anesthetized animals from the Abdominal aorta for the following investigations like Haematology and Biochemical analysis. Gross pathological changes were monitored in all the organs then the vital organs were preserved and subjected to Histopathological examination.[5]

Collection and analyses of blood

At the end of treatment period, animals were fasted overnight, then anesthetized to collect blood samples from the abdominal aorta in two tubes: one with EDTA for haematological parameters, another one without anticoagulant and was centrifuged at 4000 rpm at 4°C for 10 minutes to obtain the serum for biochemical parameters. Blood samples of control and drug treated rats were analyzed for haemoglobin (Hb), total red blood corpuscles (RBC), white blood corpuscles (WBC) count, Platelet, Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), were calculated by auto analyzer. Serum samples of control and experimental animals were analyzed for, Bilirubin, BUN, Creatinine, Triglyceride, Total Cholesterol, HDL, LDL, VLDL, using standard methods. Activities of glutamate oxaloacetate transaminase/Aspartate aminotransferase (GOT/AST), glutamate pyruvate

transaminase/ Alanine aminotransferase (GPT/ALT) were estimated as per the colorimetric procedure.

Histopathology

The organs included liver, kidneys, spleen, brain, heart, lungs, stomach, testis and uterus of the animals were preserved, and they were subjected to Histopathological examination. Histopathological investigation of the vital organs was done. The organ pieces (3-5µm thick) of all the animals (low, mid, high) w e r e preserved and fixed in 10% formalin for 24 hrs. Samples were dehydrated in an auto technic and then cleared in benzene to remove absolute alcohol. Embedding was done by passing the cleared samples through three cups containing molten paraffin at 50^{o}C and then in a cubical block of paraffin made by the "L" molds. It was followed by microtome and the slides were Prepared then stained with Haematoxylin-eosin.

Statistical analysis

Findings such as body weight changes, food consumption, water intake, and hematology and biochemical analysis were subjected to One-way ANOVA Dunnet's test using a computer software program followed by D *Graph Pad Instat-3.*

RESULTS AND DISCUSSION

In the Acute oral toxicity study, there was no abnormal signs and behavioural changes in all animals at the dose level of 300, 2000 mg/kg body weight administered orally, during the study period. There was no mortality observed after dosing of Rasa Karpoora Kuligai upto 2000mg/kg body weight This indicates that LD50 of Rasa Karpoora Kuligai is more than 2000mg/kg b.wt. There were no changes in skin and fur, eyes and mucous membranes of all animals. The eating, drinking habit, sleep pattern and locomotion were normal in all animals and no changes in body weight as compared to control group. At the end of the 14th day, necropsy was performed and there was no abnormality seen in test groups as compared to control group during the examination.

In Repeated dose oral toxicity study, all animals in this study were free of toxic clinical signs throughout the dosing period of 28 days. All animals in control and in all the treated dose groups survived throughout the dosing period of 28 days. Results of body weight determination of animals (Table No: 1) from control and different dose groups exhibited comparable body weight gain throughout the dosing period of 28 days. During dosing and the post-dosing recovery period, the quantity of food consumed by animals from different dose

groups was found to be comparable with that by control animals. Comparison of organ weights of treated animals with respective control animals on day 29 was found to be comparable similarly (Table No. 2). The results of hematological investigations (Table No. 3) conducted on day 29 revealed following significant changes in the values of different parameters investigated when compared with those of respective controls; however, the increase or decrease in the values obtained was within normal biological and laboratory limits or the effect was not dose dependent. Haematological analysis conducted at the end of the dosing period on day 29, revealed no abnormalities attributable to the treatment. Results of Biochemical investigations conducted on days 29 revealed the following significant changes in the values of hepatic serum enzymes studied. Biochemical analysis conducted at the end of the dosing period on day 29 no abnormalities attributable to the treatment. When compared with those of respective control. (Table. No. 4) However, the increase or decrease in the values obtained was within normal biological and laboratory limits. All the animals from control and all the treated dose groups up to 600 mg/kg survived throughout the dosing period of 28 days. No signs of toxicity were observed in animals from different dose groups during the dosing period of 28 days. Food consumption of control and treated animals was found to be comparable throughout the dosing period of 28 days. Histopathological examination of Organs such as brain, heart, lung, stomach, liver, kidney, thymus, spleen, testis, and ovary revealed that there were no abnormalities. (Figures 1,2,3,4,5,6,7,8,9,10).

Table No. 1: Body weight changes of test animals in 28 days repeated oral toxicity study of Rasa Karpoora Kuligai.

GROUP	CONTROL	RKK. LOW DOSE 230mg/kg	RKK.MID DOSE 450mg/kg	RKK.HIGH DOSE600mg/kg
0,DAY	126.667±1.42984	117.5±0.718795	98.3333±0.954521	95.8333±1.7966
7th DAY	132.667±1.42984	123.5±0.921955	104±1.06458	101.667±1.72562
14th DAY	138.5±1.40831	131±1.41421	110.167±1.27584	107.333±1.7062
21st DAY	144.5±1.40831	137±1.52753	116±1.18322	113±1.59165
28th DAY	150.333±1.52023	143.5±1.82117	121.667±1.22927	118.667±1.68655

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(N=10); ^{ns}p>0.05, *p<0.05, **p<0.01, ***p<0.001, calculated by comparing treated groups with control group.

Table No. 2: Organ weight changes in grams of test animals in 28 days repeated oral toxicity study of Rasa Karpoora Kuligai.

GROUP		CONTROL	RKK. LOW DOSE 230mg/kg	RKK.MID DOSE 450mg/kg	RKK. HIGH DOSE 600mg/kg
BRAIN		1.256±0.2052	1.232±0.1547	1.499±0.2023	1.159±0.2025
HEART		0.8727±0.04385	0.8323±0.06207	0.738±0.03329	0.6877±0.02028
LIVER		6.019±0.359	8.448±0.652	6.706±0.07517	5.779±0.338
LUNGS		1.447±0.1242	0.9353±0.03152	1.671±0.1854	1.278±0.2453
KIDNEY	L	0.7613±0.03038	0.8533±0.06263	0.8533±0.05191	0.7467±0.02533
	R	0.7453±0.03374	0.8287±0.06534	0.721±0.01721	0.7333±0.02809
TESTIS		3.095±0.1521	2.517±0.1654	3.295±0.08581	3.11±0.1176
UTERUS		0.736±0.02501	0.9607±0.05447	0.985±0.03436	1.214±0.306

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(N=10); $^{ns}p>0.05$, *p<0.05, **p<0.01, ***p<0.001, calculated by comparing treated groups with control group.

Table No. 3: Effect of 28 days repeated dose of Rasa Karpoora Kuligai on Haematological parameters.

GROUP	CONTROL	RKK. LOW DOSE 230mg/kg	RKK.MID DOSE 450mg/kg	RKK. HIGH DOSE 600mg/kg
RBC (X10^6/µL)	4.573±0.1139	5.39±0.3035	4.853±0.6894	5.8±0.3617
WBC(X10^3/µL)	12.5±1.531	11.47±0.5783	13±1.007	11.43±0.3756
HB (g/dl)	10.5±0.5859	13.2±0.8963	11.83±1.683	14.03±0.809
PCV %	32.2±1.833	40.6±2.689	36.17±4.725	43.1±2.427
POLYMORPHS (%)	7.333±0.6667	10±1	8±3.055	7±1.155
LYMPHOCYTES (%)	85.33±2.028	81.33±1.856	79.33±3.528	85.67±2.963
MONOCYTES (%)	5±0.5774	3±0.5774	3.333±0.6667	3.333±0.3333
EOSINOPHILS (%)	3.333±0.3333	4.333±0.3333	4.667±0.8819	4.333±0.3333
MCH (Pg)	23.6±0.611	24.93±0.4702	24.83±0.6566	25.77±0.3283

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(N=10); p>0.05, *p<0.05, **p<0.01, ***p<0.001, calculated by comparing treated groups with control group.

Table No. 4: Effect of 28 days repeated dose of Rasa Karpoora Kuligai On Biochemical parameters.

GROUP	CONTROL	RKK. LOW DOSE 230mg/kg	RKK.MID DOSE 450mg/kg	RKK. HIGH DOSE 600mg/kg
SGOT(IU/ml)	97.2±6.835	116.1±11.08	98.37±10.16	91.5±3.523
SGPT (IU/ml)	44.77±8.151	90.73±17.71	45.3±5.852	48.6±6.012
ALP (IU/ml)	210.1±69.74	175.9±11.87	169.9±27.35	186.6±24.95
TOTAL BILIRUBIN (mg/dl)	1.303±0.2452	1.453±0.2822	0.8193±0.3371	0.99±0.194
CREATININE (mg/dl)	0.6133±0.03844	0.58±0.08327	0.4767±0.0441	0.5867±0.02028
URIC ACID (mg/dl)	1.747±0.2761	1.74±0.155	2.06±0.6612	1.983±0.2924

Values are expressed as mean ± SEM Statistical significance (p) calculated by one way ANOVA followed by dunnett's(N=10); $^{ns}p>0.05$, *p<0.05, **p<0.01, ***p<0.001, calculated by comparing treated groups with control group.

Histopathology of Brain

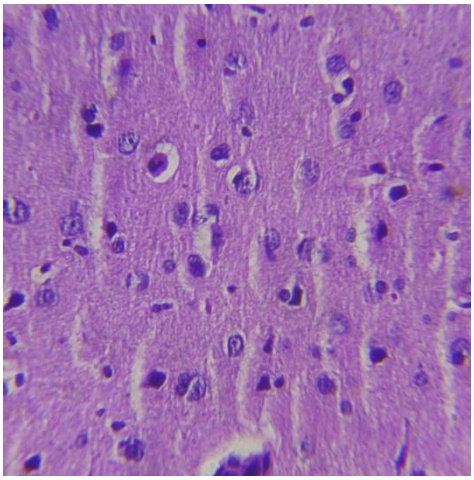

Plate A: Control Male

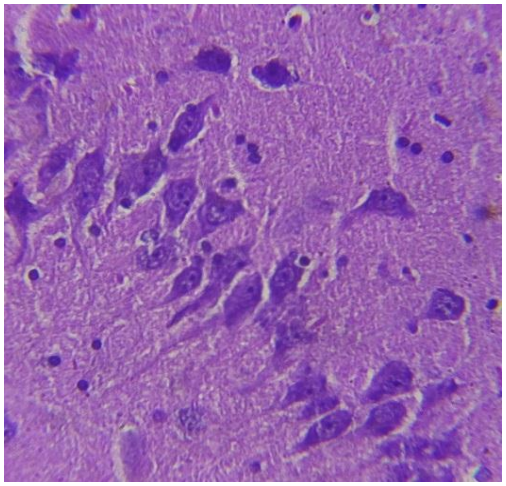

Plate B: Control Female

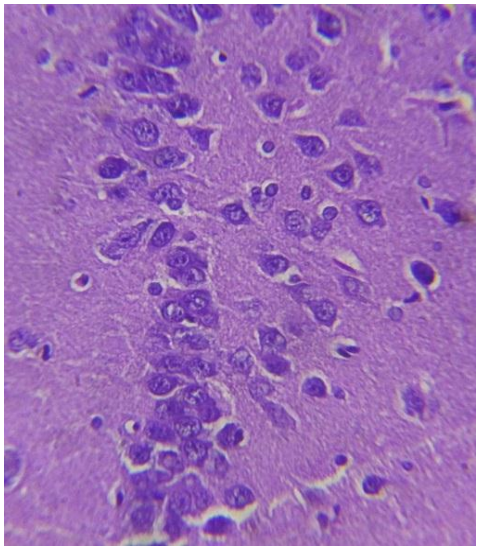

Plate C: High Dose Male

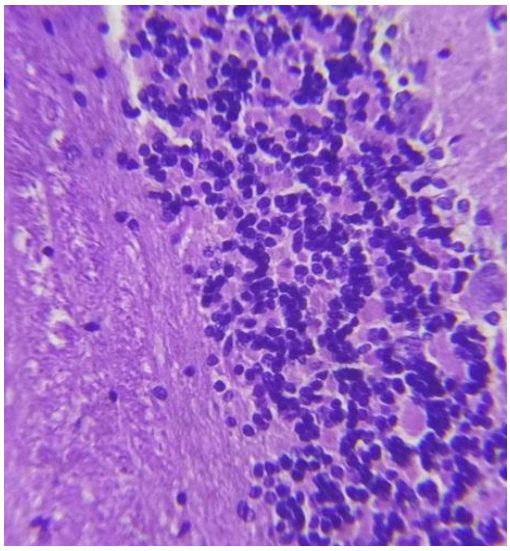

Plate D: High Dose Female

Figure 1

Plate A: Regular marginal alignment on the neurons with promising histology was observed.

Plate B: The CA zones of brain hippocampi are filled with densely packed Pyramidal cells.

Plate C: Arrangement of neurons on cerebral cortex appears normal and dense.

Plate D: Three layers of cerebellar cortex, the molecular, purkinje and granular layers, appeared clear and distinct without any changes in their cells

Histopathology of Heart

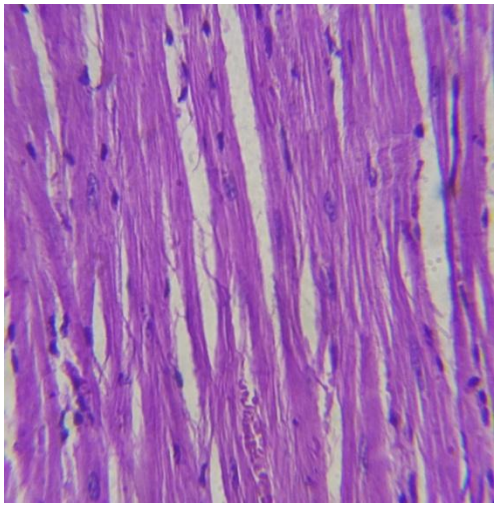

Plate A: Control Male

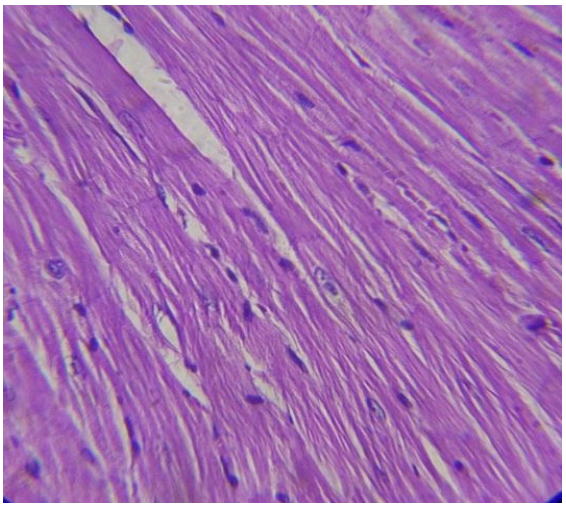

Plate B: Control Female

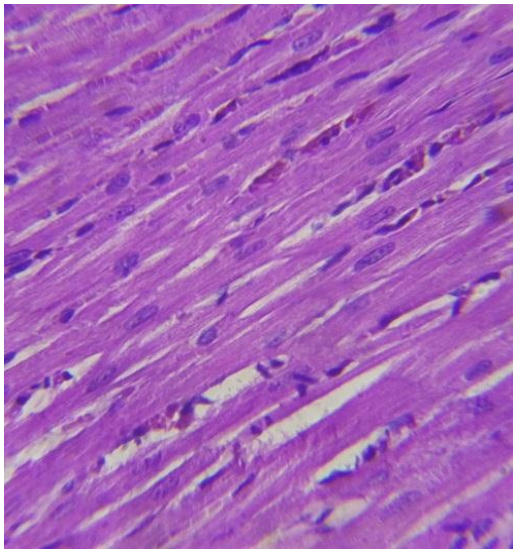

Plate C: High Dose Male

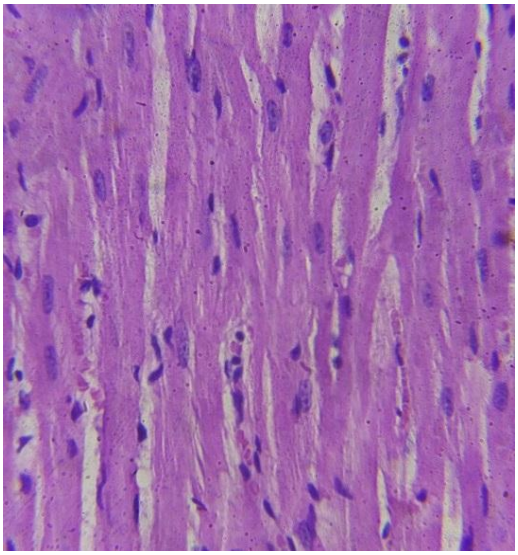

Plate D: High Dose Female

Figure 2

Plate A: Nucleus appears prominent with regular arrangement of fibres. No evidence of pyknotic nucleus.

Plate B: Myocardial cells appears normal with well-defined mycoplasma and prominent nucleus and nucleolus.

Plate C: Appearance of fibrils and cross striations are normal and equidistant.

Plate D: Appearance of cardiac myocyte was normal.

Histopathology of Lung

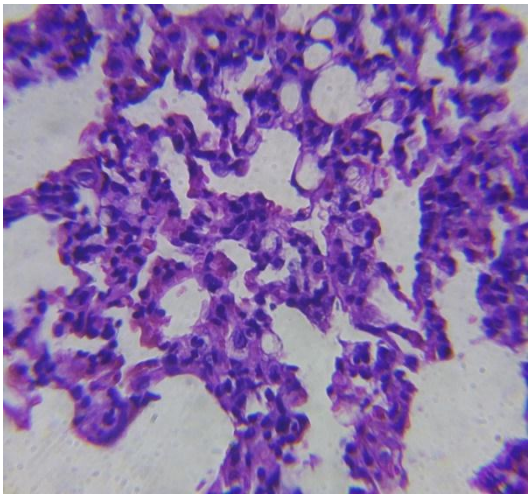

Plate A: Control Male

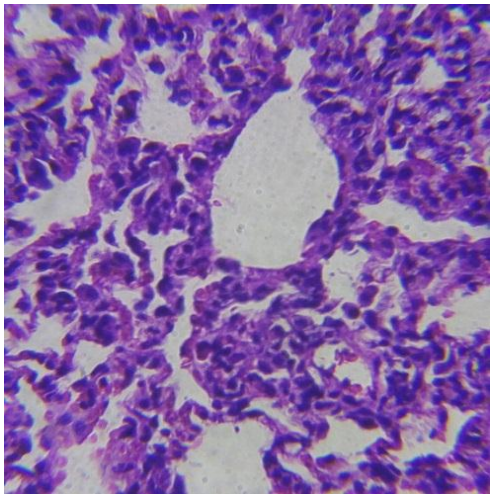

Plate B: Control Female

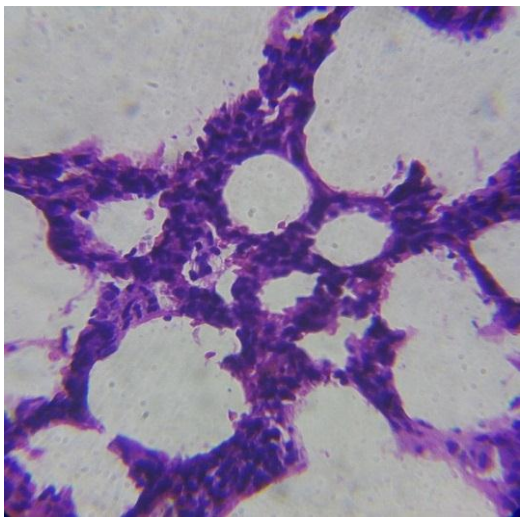

Plate C: High Dose Male

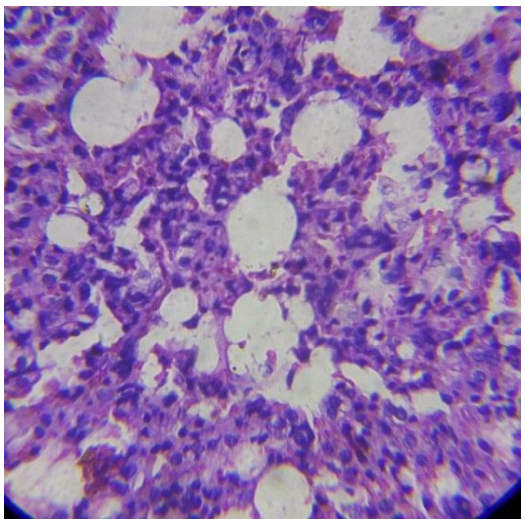

Plate D: High Dose Female

Figure 3

Plate A: Arrangement of epithelial and muscular appears normal.

Plate B: Lung parenchyma appears normal with regular arrangement of alveoli and alveolar sac with no signs of lymphocyte infiltration and pulmonary fibrosis.

Plate C: Perfect network of simple squamous epithelium were observed. Inter alveolar septum and alveolar capillary appears normal.

Plate D: Pneuomocyte and capillary appears normal.

Histopathology of Stomach

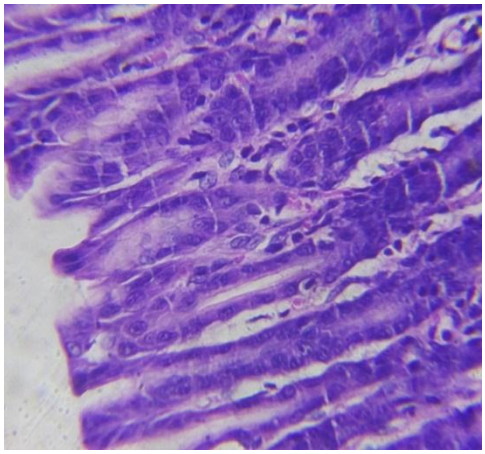

Plate A: Control Male

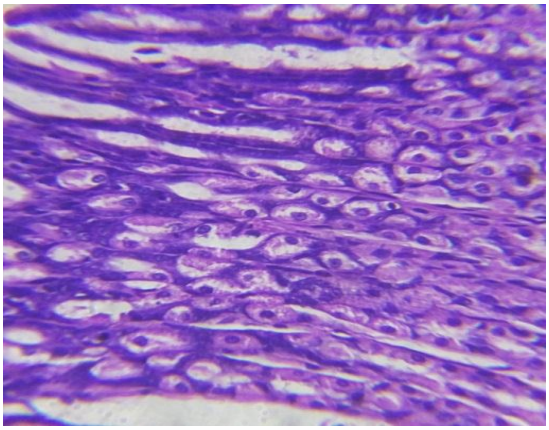

Plate B: Control Female

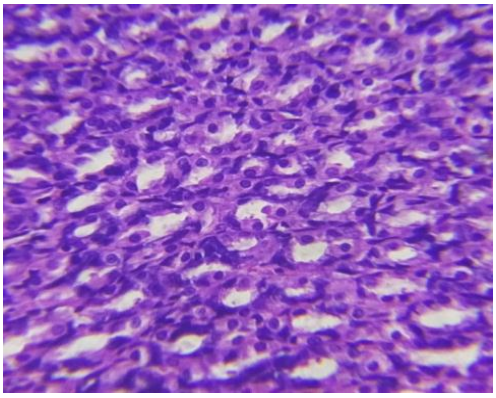

Plate C: High Dose Male

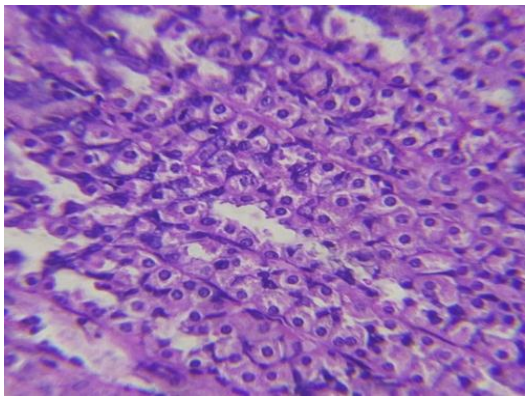

Plate D: High Dose Female

Figure 4

Plate A: Lumina of blood vessels appears normal. Appearance of glandular lumen was normal

Plate B: Regular arrangement of muscularis externa and outer longitudinal muscle were observed.

Plate C: Gastric glands including secretary sheath appears normal.

Plate D: Normal gastric mucosa containing intact gastric gland cells, parietal cells which are spherical cell with deeply stained dark nucleus.

Histopathology of Liver

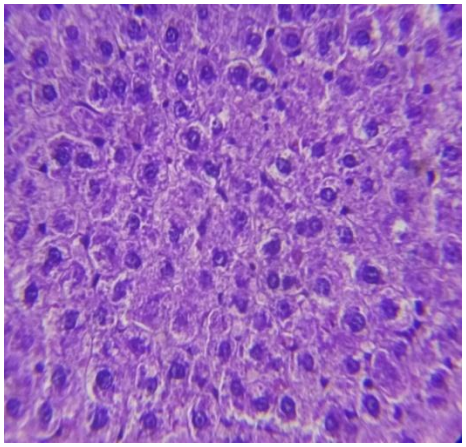

Plate A: Control Male

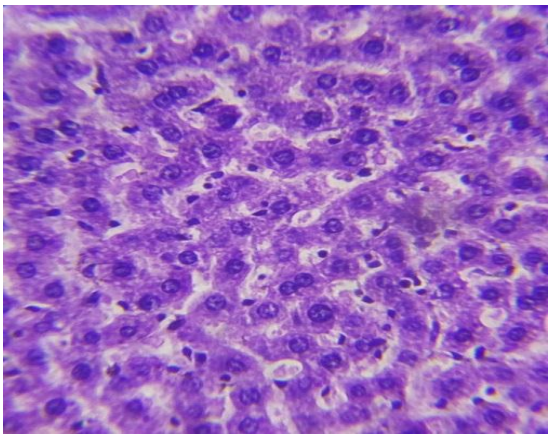

Plate B: Control Female

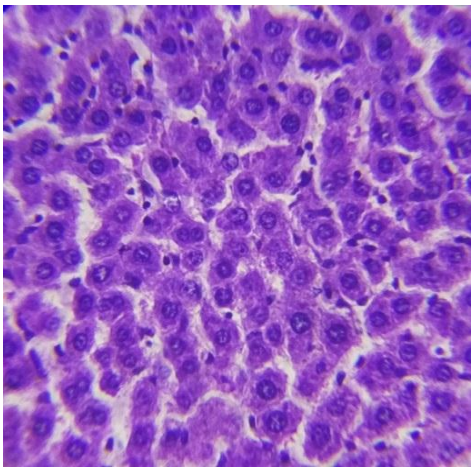

Plate C: High Dose Male

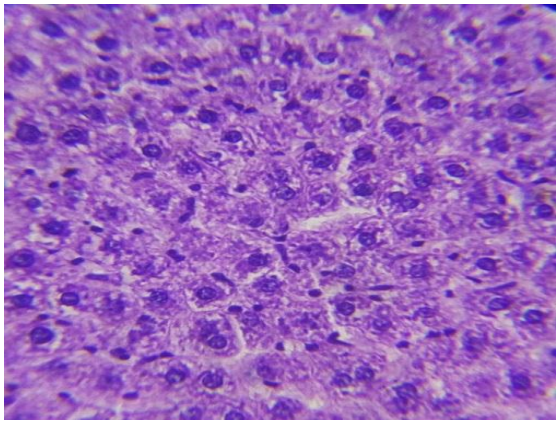

Plate D: High Dose Female

Figure 5

Plate A: Cytoplasm appears normal with widen portal tract.No signs of nodular degeneration and cirrhosis.

Plate B: The walls of the lumen appears normal with no evidence of ischemic changes.

Plate C: Liver parenchyma appears normal with no evidence of necrosis. Rare appearance of Kupffer cells with no evidence of phagocytosis in intracytoplasmic region.

Plate D: The centrilobular hepatocytes appears normal with stained cytoplasm.

Histopathology of Kidney

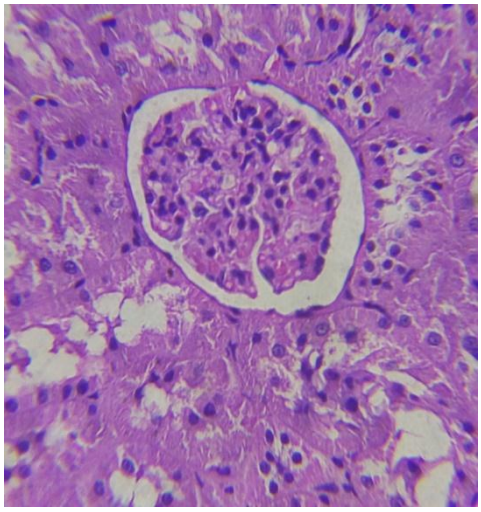

Plate A: Control Male

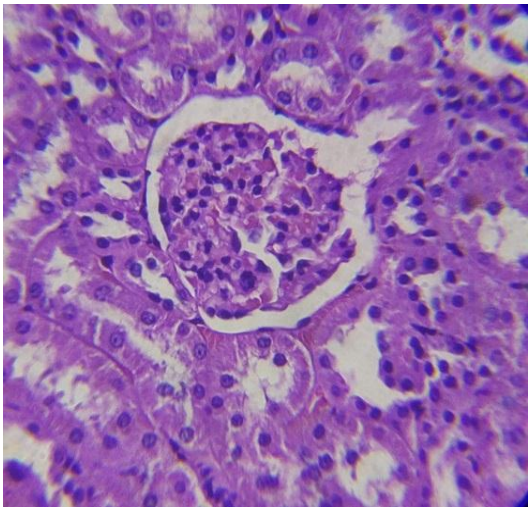

Plate B: Control Female

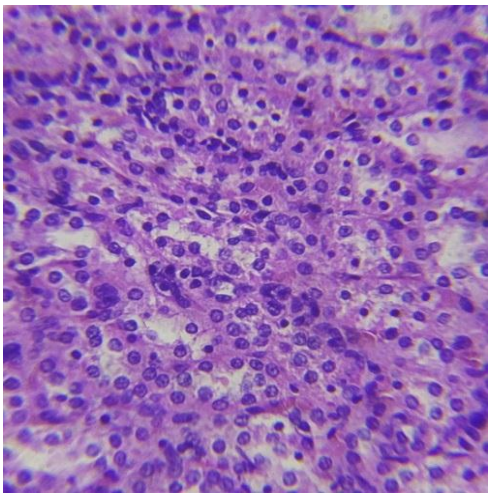

Plate C: High Dose Male

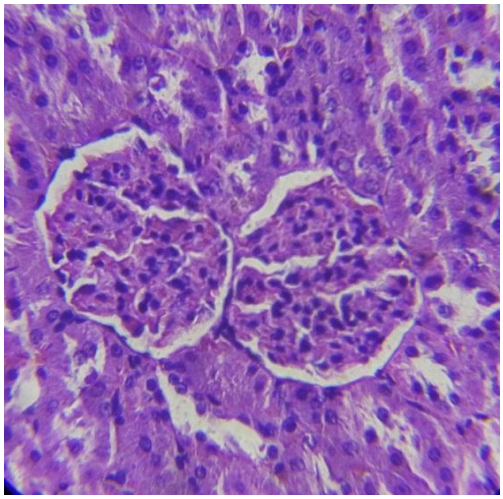

Plate D: High Dose Female

Figure 6

Plate A: Appearance of central artery and marginal sinus are normal. No abnormalities found in lymph node

Plate B: Appearance of glomerular basement membrane was normal.

Plate C: Foot processes of podocytes are separated from one another by a regular narrow Filtration Slit.

Plate D: Bowman's capsule appears normal and surrounded with Proximal Convoluted Tubule, Distal Convoluted Tubule and Collecting Duct.

Histopathology of Spleen

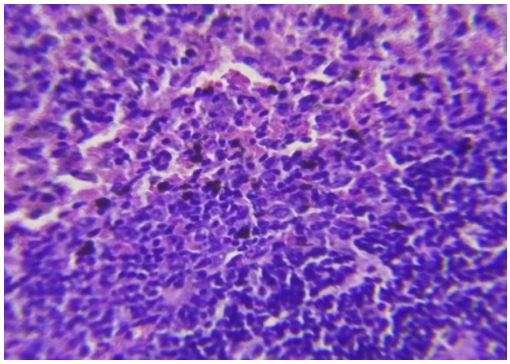

Plate A: Control Male

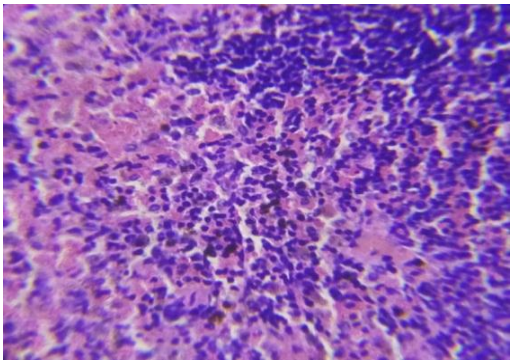

Plate B: Control Female

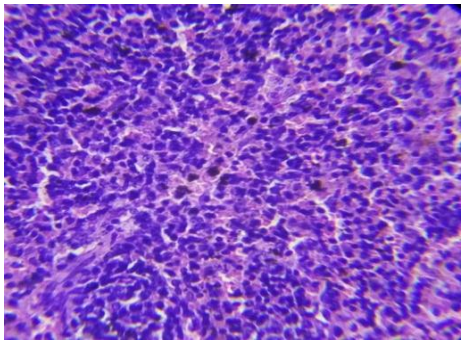

Plate C: High Dose Male

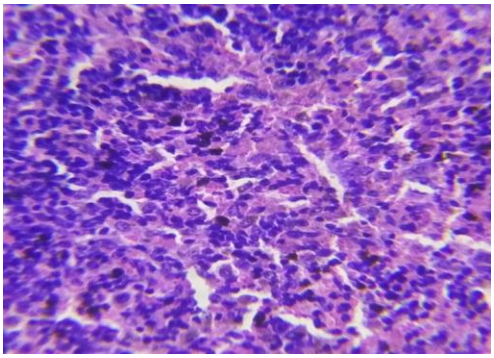

Plate D: High Dose Female

Figure 7

Plate A: Normal renal structure with rounded renal corpuscles formed of the Glomerulus. Increased bowman space around glomeruli

Plate B: Marginal vascular zone radiated in between red and white pulp. Appearance of splenic red pulp was normal.

Plate C: Lymphoid follicles appears normal.

Plate D: Erythropoietic cells (EP) are scattered throughout the red pulp with increased number of megakaryocytes.

Histopathology of Testes

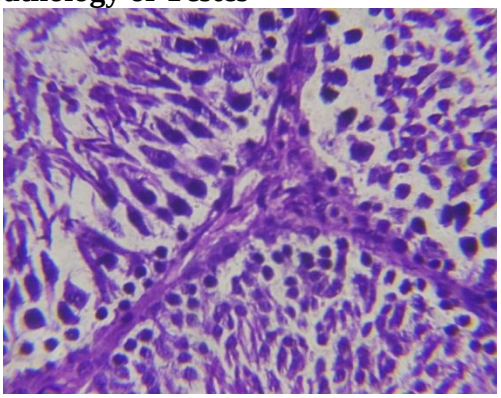

Plate A: Control Male

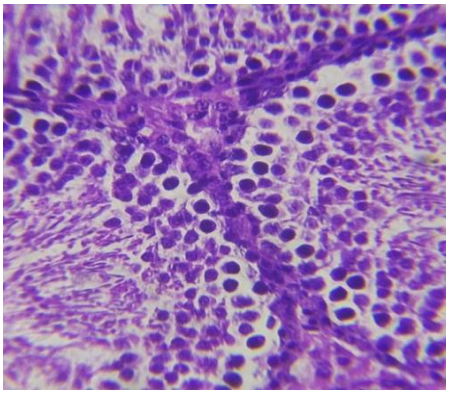

Plate B: High Dose Male

Figure 8

Plate A: Histo cytology of testicular tissue shows well differentiated germ cells with respect of spermatogonia includes spermatid and sperm were observed.

Plate B: Normal sertoli cell aligned properly on the basement membrane with oval dome shaped nucleus.

Histopathology of Uterus

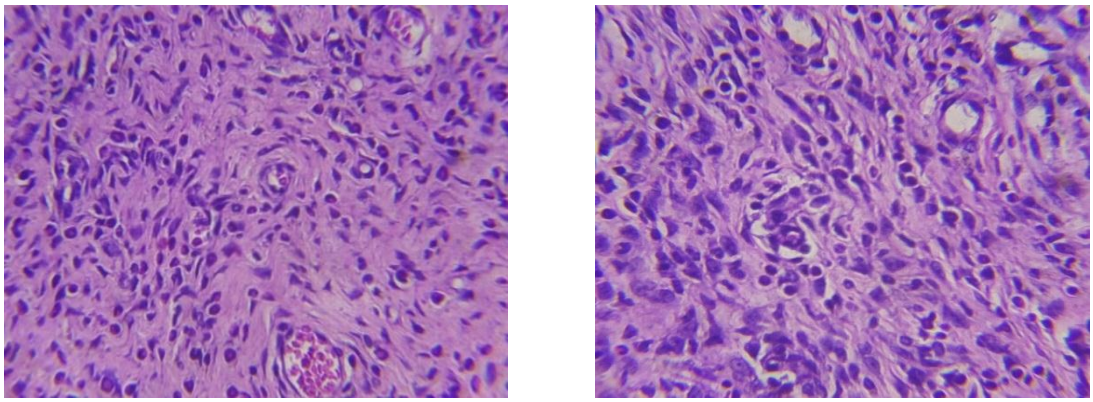

Plate A: Control Female **Plate B: High Dose Female.**

Figure 9

Plate A: Appearance of endometrium, myometrium and uterine glands was normal. **Plate B**: Endometrial stroma; G, gland; M, myometrium; P, perimetrium; L, lumen exhibits normal histological aspect of endometrium and myometrium

Histopathology of Ovary

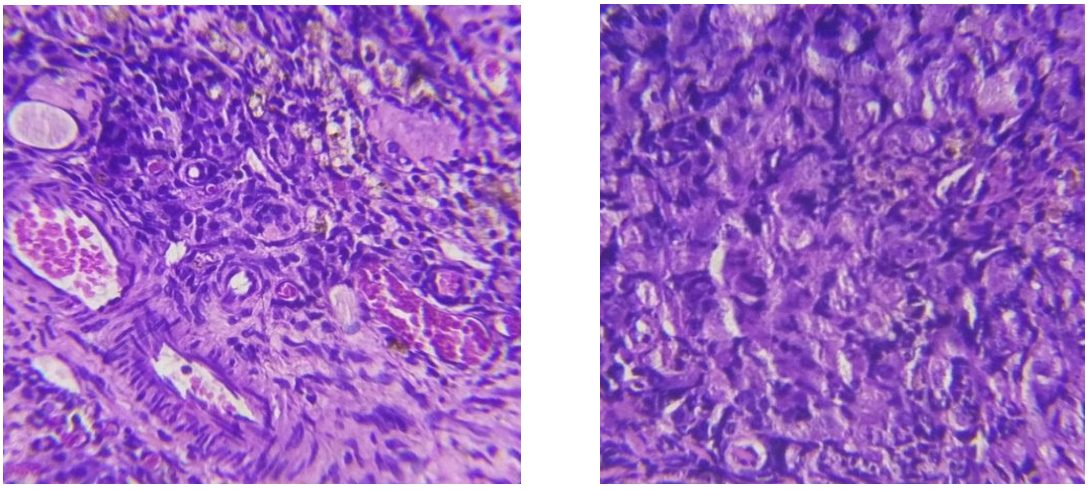

Plate A: Control Female **Plate B: High Dose Female**

Figure 10

Plate A: Follicular cells, cytoplasm and nucleus appear normal.

Plate B: Corpora lutea , atretic follicles and interstitial tissue appears normal.

CONCLUSION

From the results of analytical evaluation of the test drug *Rasa Karpoora Kuligai,* it is inferred that quality and stability was good when prepared under the standard protocol mentioned in this study. Qualitative analysis of RKK reveals the purity and bioavailability of the drug. As heavy metals were found to be with in the permissible limit, so the drug is safe for oral consumption. The particle size of the test drug was determined by SEM analysis. In vivo toxicity study reveals the drug RKK shows no mortality and signs of toxicity upto 2000 mg/Kg bodyweight in acute oral administration. In 28 days repeated oral toxicity study there was significantly changes in haematological, biochemical parameter in RKK (230mg, 450mg &600mg /Kg bodyweight) treated group but the levels were with in physiological limits. The histopathology report also confirms that there were no remarkable cellular changes at all the dose level. Based on the results, it can be concluded that, the dose level of *Rasa Karpoora Kuligai*, at *sundai alavu* (0.798mg) mentioned in G*unapadam Thathu Jeevam* is a safe dosage for human consumption.

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Conflict of interest

The authors declare that there is no conflict of interests.

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